Short Communication

Determination of Angiogenic Factors in Serum by Protein Array in Patients with Renal Cell Carcinoma

(renal cell carcinoma / angiogenin / PDGF / MCP-1 / protein array method)

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Abstract. Using the protein array method we determined the serum levels of a number of angiogenic factors. We identified serum levels of angiogenin, PDGF and MCP-1 (CCL2 chemokine) in serum of 32 patients with RCC, and 14 healthy volunteers by means of antibody array analysis. The patients were divided into three groups according to their disease stages (I+II, III, and IV). We found significant differences between the controls and patients with RCC both pre-operatively and post-operatively in angiogenin, PDGF and MCP-1 serum levels. The increase in angiogenin, PDGF and MCP-1 lasted in patients with RCC stages I-III even without metastases eight weeks post-operatively. The patients with stage IV RCC showed disturbed production of PDGF and MCP-1. Protein array analysis is a powerful tool for the identification of large numbers of trace proteins. Multiplex antibody array is able to provide data more precisely reflecting the nature of pathological processes.

Introduction

Under the physiological conditions angiogenesis is regulated by the balance between pro-angiogenic and anti-angiogenic factors. Several methods of proteomic analysis have been explored and used for the detection of these low-abundance proteins (Glasson et al., 1998; Cook et al., 2001; Herber et al., 2001). The antibody (protein) array technology offers an alternative approach to obtaining proteomic data on dozens of cytokines, chemokines, growth factors, angiogenic modulators, and other proteins. Antibody arrays are suitable for identification and determination of the relative distribution of potential biomarkers in biological fluids. In this role, the arrays have enormous potential. Once established, membrane-bound antibody arrays are inexpensive and can be easily constructed in a laboratory. They can be performed with minimum laboratory facilities by individuals without extensive laboratory training and without the need to invest in expensive equipment.

Angiogenic factors play a critical role in the growth and progression of tumours.

Hypoxia of tumour cells initiates transcription of the angiogenin gene. Angiogenin is a polypeptide of enzymatic nature which stimulates endothelial cells to produce prostacyclin (by activating phospholipase C and phospholipase A_2) and inhibits production of adhesion molecules ICAM-1 (intracellular adhesion molecule 1) and VCAM-1 (vascular adhesion molecule 1). The results of studies suggest that under specific conditions, endothelial cells express an angiogenin receptor that may mediate angiogenin-stimulated DNA synthesis and proliferation and play an important role in angiogenin-induced angiogenesis (Hu et al., 1997). Because of these properties, angiogenin has been considered to be a useful marker in the monitoring of tumour treatment and detection of tumour recurrence (Distler et al., 2003; Pavel et al., 2005).

A number of clinical studies suggest that angiogenin is closely related to tumour growth and progression. Increased levels of angiogenin were found in various solid tumours; higher levels were associated with poor prognosis. However, the results of all studies are not homogenous. For example, two studies in breast cancer show

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Abbreviations: bFGF – basic fibroblast growth factor, MCP-1 – monocyte chemo-attractant protein 1, PDGF – platelet-derived growth factor, RCC – renal cell carcinoma, VEGF – vascular endothelial growth factor.

opposite results; elevated aniogenin levels were associated with a good prognosis in one study (Sheen-Chen et al., 2000) and a poor prognosis in another (Eppenberger et al, 1998).

Tumour angiogenesis is also regulated by other pro-angiogenic factors, above all, soluble factors that are produced by the tumour, stromal and endothelial cells. Chemokines play an important role during angiogenesis as well. Monocyte chemo-attractant protein 1 (MCP-1) is a member of the CC chemokine family and plays a key role during the monocyte activation and tumour stroma infiltration by monocytes/macrophages (Ueno et al., 2000; Hemmerlein et al., 2001). Tumour-associated magrophages (TAMs) participate in creation of the tumour microenvironment, which is decisive for neoangiogenesis. Many studies show that the tumour infiltration by macrophages has a negative effect on patients' survival.

The tube formation of new vessels is regulated by platelet-derived growth factor (PDGF) (Xu et al., 2006). PDGF is a mitogen that stimulates proliferation of fibroblasts and increases collagen production. It also acts as a chemo-attractant for endothelial cells. PDGF and its receptors are homodimeric and heterodimeric proteins containing two α and β chains. Endothelial cells are expressed only on the PDGF-β receptor. PDGF plays an irreplaceable role during the vessel stabilization (Distler et al., 2003). PDGF was originally isolated from blood platelets (Heldin et al., 1979). Later studies showed that it is synthesized by other, e.g. epithelial and endothelial cells, fibroblasts, keratinocytes and macrophages (Lindroos et al., 1997; Demayo et al., 2002). It binds to the PDGFR receptor from the family of tyrosine kinase receptors. PDGF also shows a chemo-attractive effect on neutrophils and monocytes. TAMs together with endothelial cells are an important source for PDGF synthesis, which plays a key role in oncogenesis and anti-tumour immune responses (Li et al., 2007). The expression of PDGFR-α may be related to the duration of patients' survival (Tawfik et al., 2007) and it correlates with tumour progression (Sulzbacher et al., 2003).

For detection of angiogenic factors we used antibody array, which is capable of detecting up to 20 angiogenic factors in one experiment, and we analysed relative serum levels of these proteins. In this study we investigate whether the levels of serum angiogenic factors (angiogenin, MCP-1, PDGF) in patients with clear-cell renal cell carcinoma (RCC) are elevated in comparison with healthy controls and correlate with the disease stage.

Table 1. Clinical characteristics of 32 patients with RCC

Patient	Sex	Age	Stage	Tumour	Nodes	Metastasis	Grade	Location, penetration and number of metastatic lesions
1	f	78	I	T1a	N0	M0	II	none
2	m	74	I	T1b	N0	M0	II	none
3	m	80	I	T1b	N0	M0	II	none
4	m	55	I	T1b	N0	M0	I-II	none
5	m	55	I	T1a	N0	M0	II	none
6	m	59	I	T1b	N0	M0	II	none
7	m	51	I	T1b	N0	M0	III	none
8	m	78	I	T1b	N0	M0	II	none
9	m	58	I	T1a	N0	M0	III	none
10	f	72	I	T1b	N0	M0	I-II	none
11	f	46	I	T1a	N0	M0	I	none
12	m	50	II	T2	N0	M0	II	none
13	m	66	II	T2	N0	M0	II	none
14	m	54	I	T1a	N0	M0	III	none
15	m	78	I	T1a	N0	M0	II	none
16	f	83	III	T3a	N0	M0	I-II	adrenal
17	m	66	III	T3b	N0	M0	II	renal vein
18	f	73	III	T3b	Nx	M0	III	renal vein
19	m	64	III	T3b	N0	M0	II	renal vein
20	m	57	III	T3a	N0	M0	II-III	bilateral RCC
21	f	66	III	T3a	N0	M0	II	adrenal
22	f	77	III	T3a	N0	M0	II-III	adrenal
23	m	75	III	T3a	N0	M0	I-II	adrenal
24	m	77	IV	T3a	Nx	M1	III	liver (1)
25	m	74	IV	T4	N0	M1	III-IV	skeleton, liver, lung (3)
26	f	70	IV	T3b	N2	M0	III-IV	lymph nodes (1)
27	f	71	IV	T3a	N0	M1	III	liver, peritoneum (2)
28	f	62	IV	T1b	N0	M1	I-II	lymph nodes - mediastinum (1)
29	m	44	IV	T3b	N0	M1	III-IV	liver, lung (2)
30	f	74	IV	T1b	Nx	M1	III	lung (1)
31	m	57	IV	T3b	N0	M1	II	skeletal multipath (3)
32	m	65	IV	T1b	N1	M1	III	spine, lung (2)

Notes: f – female, m – male, stage (according to AJCC), grade (according to Fuhrman)

Material and Methods

Patients. In total 32 patients (21 males and 11 females, mean (SD) age 65.9 (10.8) years) with clear-cell RCC, diagnosed between October 2005 and September 2006, were enrolled in our study. In all patients the primary renal tumour was removed. Eight partial resections and 24 nephrectomies were carried out. The diagnosis of RCC was confirmed histologically. Grading was performed by the Fuhrman grading system and patients were divided into three groups according to the disease stages (Greene et al., 2002) (Table 1). The control group consisted of 14 healthy volunteers, eight males and six females, mean (SD) age 55.3 (11.6) years. The study was approved by the institutional ethics committee of the Teaching Hospital. All patients provided written informed consent.

Sera samples. Patient sera were obtained by repeated peripheral venous blood collections, which were carried out on the day of surgery, day 7, and eight weeks post-operatively. One hour after blood collection, each blood sample was centrifuged at 800 x *g* for 10 min. Sera were subsequently divided into two parts and stored at - 20 °C until the time of processing. Control sera were obtained from 14 healthy blood donors of similar age.

Protein array method. In order to determine the level of angiogenic factors, the antibody array method by RayBiotech Company (Norcross, GA), RayBio Human Angiogenesis Antibody Array I, was used. This membrane contains positive controls (3 x 2 spots), negative controls (2 x 2 spots) and 20 x 2 spots for determining angiogenin levels, endothelial growth factor-78 (ENA-78), basic fibroblast growth factor (bFGF), pangene-related oncogene (pan-GRO), interferon γ (IFN-γ), insulin-like growth factor 1 (IGF-1), interleukin 6 (IL-6), interleukin 8 (IL-8), leptin, MCP-1, PDGF-BB, placental growth factor (PIGF), regulated on activation, normal T-expressed and secreted (RANTES), transforming growth factor β1 (TGF-β1), tissue inhibitor metalloproteinases 1, 2 (TIMP-1, TIMP-2), thrombopoietin and vascular endothelial growth factor (VEGF-D). The detection of the above-mentioned proteins was carried out according to the User Manual (revised May 28, 2004) RayBio Human Cytokine Antibody Array (www.raybiotech.com). The immunocomplexes that were formed and fixed in the area of appropriate spots were visualized by peroxidase-conjugated streptavidin. The result is a membrane with visible spots with varied intensity. The concentration of an appropriate factor then complies with the intensity of colouring of a particular spot evaluated densitometrically. The evaluation was carried out by means of ARES ARay Evaluation System software (Baria, Psáry-Dolní Jirčany, Czech Republic). The resulting concentration of individual proteins was expressed as relative intensity (value) of spot colouring in comparison to controls.

Statistical analysis. Statistical evaluation of the measured values was carried out by means of NCSS 2007 Statistica. Differences between the groups were analysed by Mann-Whitney U-test, Kolmogorov-Smirnov test and ANOVA analysis with Fisher test. The data are expressed as mean ± standard deviation. The differences were considered to be significant when $P < 0.05$.

Results and Discussion

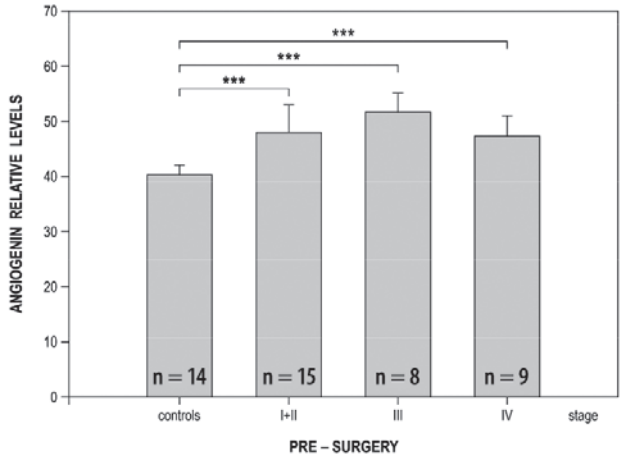
We compared the levels of angiogenin, PDGF and MCP-1 in serum of patients with RCC and healthy controls.

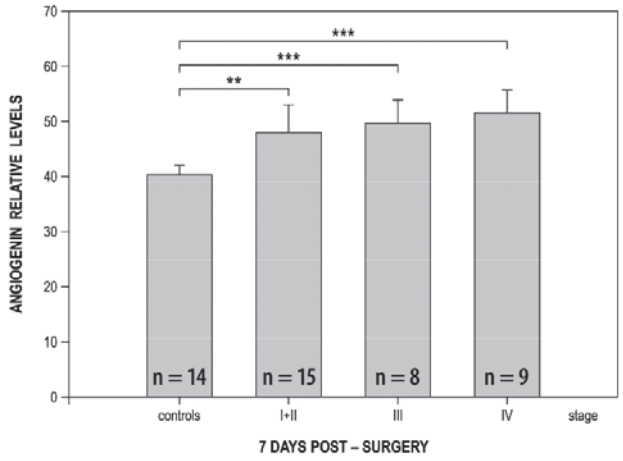
Serum levels of angiogenin were significantly higher pre-operatively in patients in all stages of RCC in comparison to healthy blood donors. All patients showed a similar increase in serum angiogenin levels and the elevated levels persisted after tumour removal in all stages (see Table 2 and Fig. 1)

Table 2. Comparison of relative levels of angiogenin, PDGF and MCP-1 in samples pre-operatively, seven days and eight weeks post-operatively.

	control (N = 14)	stage I + II	stage III	stage IV	control v. I+II	control v. III	control v. IV	I+II v. III	I+II v. IV	III v. IV
Angiogenin										
sample 1	40.44 ± 1.62	48.13 ± 5.12	51.94 ± 3.39	47.57 ± 3.73	P < 0.001	P < 0.001	P < 0.001	n.s.	n.s.	n.s.
sample 2		48.50 ± 6.36	50.25 ± 4.11	52.20 ± 3.68	P = 0.002	P < 0.001	P < 0.001	n.s.	n.s.	n.s.
sample 3		48.13 ± 4.97	50.67 ± 2.46	48.90 ± 4.45	P < 0.001	P < 0.001	P < 0.001	n.s.	n.s.	n.s.
PDGF										
sample 1	39.11 ± 2.39	44.08 ± 5.37	42.94 ± 3.99	35.45 ± 3.96	P = 0.004	P = 0.005	P = 0.006	n.s.	P < 0.001	P = 0.004
sample 2		45.73 ± 6.48	44.87 ± 2.63	40.47 ± 7.05	P = 0.002	P < 0.001	n.s.	n.s.	P = 0.020	n.s.
sample 3		45.17 ± 5.51	44.01 ± 2.40	36.11 ± 5.65	P < 0.001	P < 0.001	n.s.	n.s.	P < 0.001	P = 0,003
MCP-1										
sample 1	9.78 ± 3.55	19.93 ± 7.92	23.06 ± 5.69	13.43 ± 4.83	P < 0.001	P < 0.001	n.s.	n.s.	P = 0.034	P = 0.007
sample 2		25.57± 7.85	25.44 ± 7.65	17.31 ± 6.25	P < 0.001	P < 0.001	P = 0.007	n.s.	P = 0.008	P = 0.022
sample 3		23.94 ± 7.28	25.49 ± 8.01	12.24 ± 6.29	P < 0.001	P < 0.001	n.s.	n.s.	P < 0.001	P < 0.001

Notes: relative concentrations are expressed as mean value ± standard deviation (SD), n.s. – no significance, P value level, v. – versus, sample 1 – collected pre-operatively, sample 2 – collected seven days post-operatively, sample 3 – collected eight weeks post-operatively





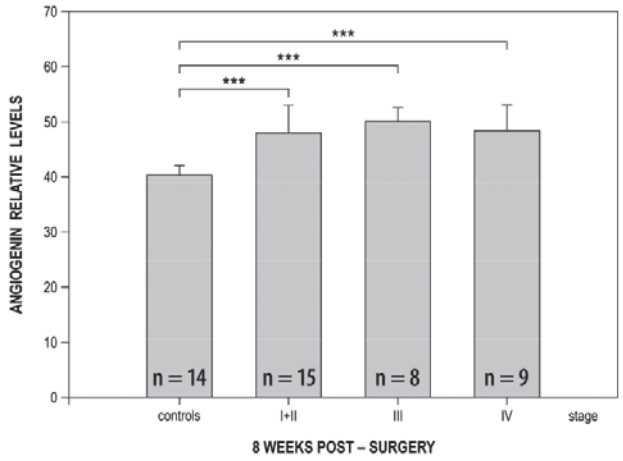
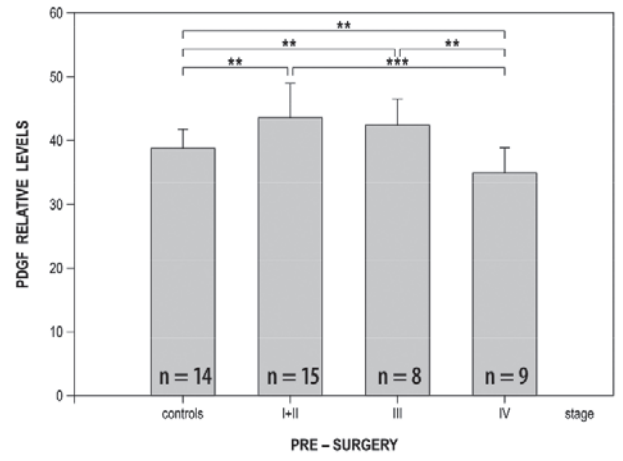
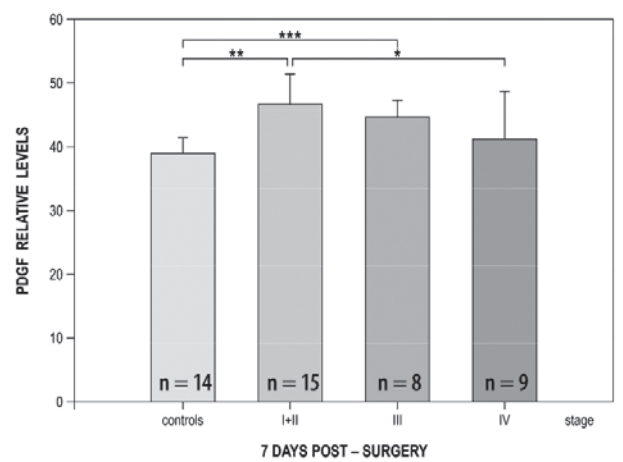


Fig. 1 Serum levels of angiogenin pre-operatively, seven days and eight weeks post-operatively. Results are shown as means ± SD of relative levels. *P < 0.05, **P < 0.01, ***P < 0.001





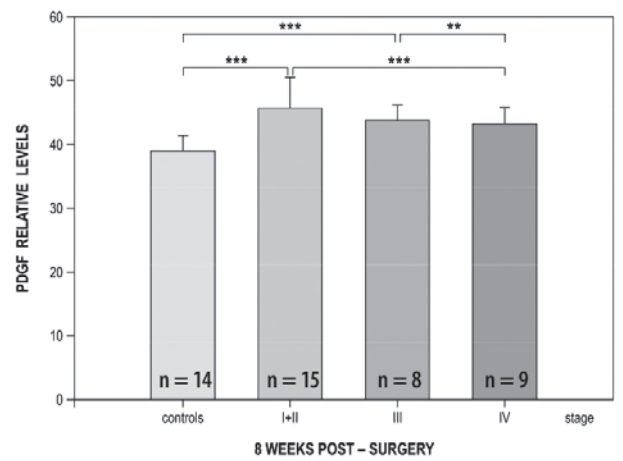


Fig. 2 Serum levels of PDGF pre-operatively, seven days and eight weeks post-operatively. Results are shown as means ± SD of relative levels. *P < 0.05, **P < 0.01, ***P < 0.001

PDGF levels in serum of patients with stages I+II and III RCC were significantly increased in comparison to the group of healthy controls. In patients with stage IV RCC, the pre-operative PDGF was significantly lower in comparison to the control group. In the course of monitoring, patients with stage IV RCC had significantly lower serum levels than those with stage I+II and III (see Table 2 and Fig. 2)

In patients with stages I+II and III RCC, the serum level of MCP-1 showed significant elevation compared to both the control group and patients with advanced RCC (stage IV). A significant increase in MCP-1 in pa-

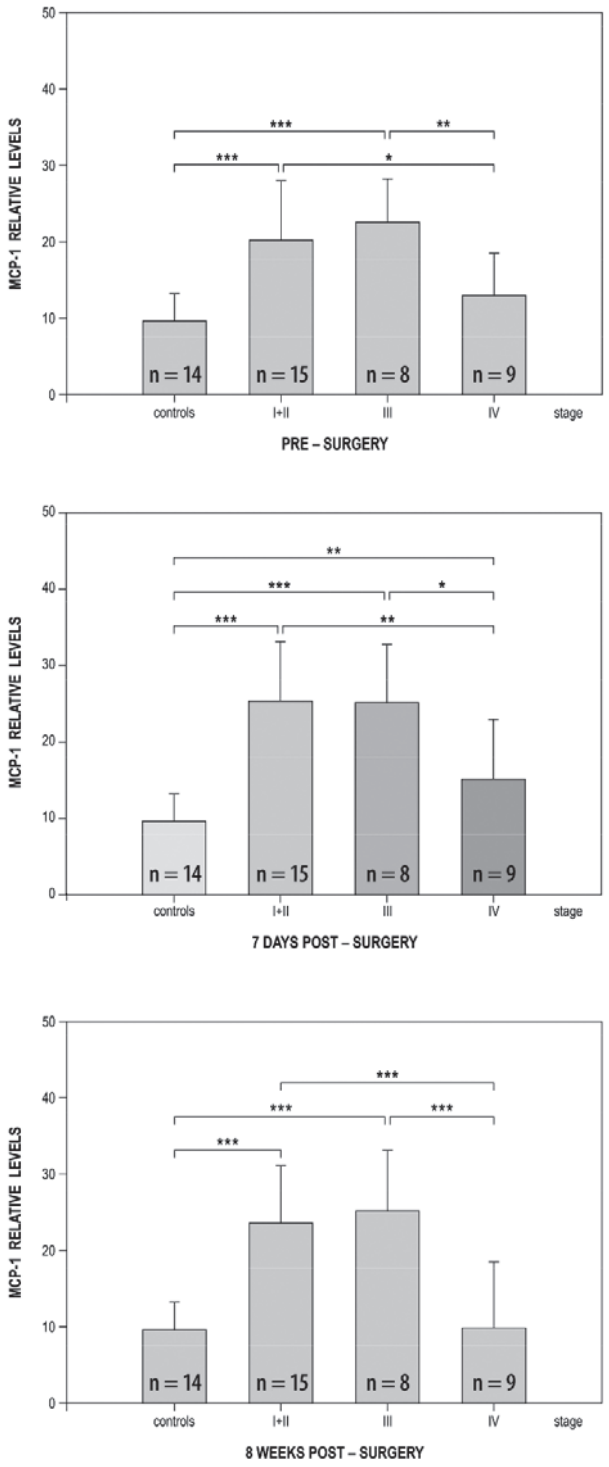


Fig. 3 Serum levels of MCP-1 pre-operatively, seven days and eight weeks post-operatively. Results are shown as means ± SD relative levels. *P < 0.05, **P < 0.01, ***P < 0.001

tients with stage IV was seen only on day 7 post-operatively (see Table 2 and Fig. 3)

The protein array method enabled us to detect serum levels of MCP-1, PDGF and angiogenin, while other factors, e.g. VEGF, bFGF and IFN-γ, were not detected by this method. There are several possible explanations for these findings. First of all, membrane protein arrays show different sensitivity limits for single factors, e.g. 3 pg/l for MCP-1, 10 pg/l for angiogenin and 10,000 pg/l for VEGf-D or bFGF. However, the different sensitivity for the evaluated proteins is probably not the only cause why some angiogenic factors were not detected in the serum of patients with RCC. Some of the assessed serum factors occur in very low levels, under the limit of detection, e.g. IFN-γ (declared limit of sensitivity 100 pg/ml). In our study we did not use the diluted testing serum to determine low concentrations of some factors, but other serum proteins might have interfered with antibodies and essentially mask the analytes from being detected (Copeland et al., 2004). This means, in practice, that the number of proteins that can be evaluated is lower than the number of trace proteins applied to the membrane (Huang et al., 2001; Sack et al., 2005).

In our study, we targeted the particular angiogenic factors – angiogenin, MCP-1 and PDGF – that showed some correlation with clinical findings. The study of Wechsel et al. (1999) did not confirm the correlation between capillary (vascular) density in tumour tissue and serum levels of VEGF, bFGF and angiogenin in patients with RCC. No correlation between VEGF and bFGF serum levels, disease stage, cellular grading and disease prognosis was observed (Edgren et al., 2001; Beecken et al., 2002). The above-mentioned findings and results in our study may be caused by the compartmentalization of production of angiogenic factors to the tumour focus remaining without systemic response (Wechsel et al., 1999). Due to higher sensitivity of endothelial cells of tumour vessels to pro-angiogenic stimuli, only a slight increase in pro-angiogenic factors can lead to their stimulation, while endothelial cells of normal vessels remain unaffected (Beecken et al., 2002). Production of inhibition factors that neutralize the systemic response may be another explanation for low levels of angiogenic factors or their lack in the serum. The neutralization may occur through competitive linking to a specific soluble receptor or proteolytic degradation. An example may be the Lewis lung tumour, where angiogenesis is intensively stimulated at the site of the primary tumour while growth of distant metastases is blocked by enzymatic degradation of plasminogen to angiostatin, which is an effective angiogenesis inhibitor (O'Reilly et al., 1994). The results of studies of tumour angiogenesis are also affected by the unstable character of some factors, which leads to the loss of their biological activities during preservation.

In patients with various types of tumours, an increase in some angiogenic factors in the serum was described (Kulbe et al., 2005). In our group of patients, an increase in angiogenin was found in patients with all stages in comparison to the control group. Moreover, the increase lasted for eight weeks following nephrectomy. The comparison of angiogenin levels preoperatively and seven days post-operatively showed a non-significant increase, which could be explained by the post-operative wound healing. A significant increase in angiogenin, however, lasted as late as eight

weeks after tumour removal both in patients with metastasizing RCC and without proved metastases. A long-term increase in angiogenic factors has been described in wound healing complicated by chronic infections or in patients with e.g. diabetes mellitus, rheumatoid arthritis, bronchial asthma, etc. (Wetzler et al., 2000; Sido et al., 2004). In our patients, the post-operative wound healing was without any complications and none of our patients suffered from chronic inflammatory disease during the time of surgery.

Tumour angiogenesis is characterized by a significant participation of innate cells, macrophages and dendritic cells (Sica et al., 2006). In patients with tumour diseases, MCP-1 is produced mainly by tumour cells. Depending on the presence of other chemokines and cytokines determining the character of tumour microenvironment, it regulates the monocyte infiltration of the tumour (Hemmerlein et al., 2001). PDGF is synthesized not only by thrombocytes, but also by other cells, e.g. endothelial cells and macrophages (Lindroos et al., 1997; Demayo et al., 2002). PDGF displays a chemo-attractive effect on neutrophils and monocytes, being thus involved in the regulating network that modulates the function of macrophages. PDGF is an important factor during the tube formation of new vessels (Ostman and Heldin, 2001; Distler et al., 2003; Stadler, 2005; Li et al., 2007).

While production of VEGF, angiogenin, bFGF and PDGF increases under the hypoxic conditions, in case of MCP-1 it is the opposite (Negus et al., 1998; Hemmerlein et al., 2001; Coussens and Werb, 2002). Significantly lower levels of MCP-1 in our patients with advanced RCC may be caused by the local hypoxia of tumour tissue, which is often elevated up to central necrosis of the tumour. TAMs were detected in immunohistochemical findings of large tumour masses even without central necrosis only on the edge of healthy and tumour tissue. They are not found in the centre of the tumour (Sica et al., 2006). Tissue hypoxia is also caused by anaemia. Insufficient vessel supply of the tumour and secondary anaemia in patients with metastasizing RCC are probably the main causes of a significant decrease in the serum level of MCP-1 in patients with stage IV RCC. In patients with stages I + II and III RCC, an increase in serum levels of MCP-1 lasted as late as day 7 ($P < 0.001$) and week 8 ($P < 0.001$) after primary tumour removal in comparison to the control group. On the contrary, at week 8 post-operatively in patients with stage IV RCC, the serum level of MCP-1 decreased, even though the distant metastases were present and angiogenin levels remained elevated. It seems that in the stage of advanced RCC, the low serum level of MCP-1 confirms the insufficient recruitment of cells of the monocyte/macrophage lineage. The recruitment of dendritic cells and monocytes/macrophages is necessary for the effective anti-tumour cytotoxic response. In oncological practice, the insufficient immune response to tumour antigens cannot be changed even by the current treatment methods (Lewis and Pollard, 2006).

Conclusion

Advances in miniaturization, rapid expansion of libraries of available matched high-affinity antibody pairs, development of new methods for antibody binding to the support and use of newly-made sensitive detection systems have revolutionized the potential of protein array analysis. These developments make it probable that arrays will be available in the near future and will allow simultaneous assay of biological fluids with hundreds of biologically important proteins. The angiogenic factors and their receptors are examples of these molecules; they are potential targets for cancer therapy. Other possibilities of targeted treatment have been explored. Mapping of chemokine and cytokine interactions in the regulating network of angiogenesis thus seems necessary. For this purpose, the multiparametric protein array analysis seems to be a method of choice.

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